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# Altered transition metal homeostasis in the cuprizone model of demyelination

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ABSTRACT

In the cuprizone model of demyelination, the neurotoxin cuprizone is fed to mice to induce a reproducible pattern of demyelination in the brain. Cuprizone is a copper chelator and it has been hypothesized that it induces a copper deficiency in the brain, which leads to demyelination. To test this hypothesis and investigate the possible role of other transition metals in the model, we fed C57Bl/6 mice a standard dose of cuprizone (0.2% dry chemical to dry food weight) for 6 weeks then measured levels of copper, manganese, iron, and zinc in regions of the brain and visceral organs. As expected, this treatment induced demyelination in the mice. We found, however, that while the treatment significantly reduced copper concentrations in the blood and liver in treated animals, there was no significant difference in concentrations in brain regions relative to control. Interestingly, cuprizone disrupted concentrations of the other transition metals in the visceral organs, with the most notable changes being decreased manganese and increased iron in the liver. In the brain, manganese concentrations were also significantly reduced in the cerebellum and striatum. These data suggest a possible role of manganese deficiency in the brain in the cuprizone model.



## 1. Introduction

The chemical cuprizone is administered to rodents to produce a model of demyelination that is widely used to study the mechanisms of myelin loss in the brain in disorders such as Multiple Sclerosis (MS) (Gudi et al., 2014). Cuprizone is a known copper chelator *in vitro* (Peterson, 1955) and causes axonal demyelination when administered to rodents through their diet; hence, it has been hypothesized that its mechanism of toxicity is an induced copper deficiency in brain tissue.

Cuprizone is commonly administered to C57BL/6 mice, as this strain seems particularly susceptible to the resulting demyelination (Skripuletz et al., 2008). Following a weeks-long exposure to cuprizone, astrogliosis and oligodendrocyte apoptosis are observed in the brain, with demyelination evident after 4–6 weeks in multiple structures (Falangola et al., 2014). This loss of myelin is most widely reported in the corpus callosum (Falangola et al., 2014; Steelman et al., 2012) but occurs in other myelinated brain areas including the striatum (Pott et al., 2009), cerebellum (Groebe et al., 2009; Skripuletz et al., 2010), hippocampus (Koutsoudaki et al., 2009) and cortex (Gudi et al., 2009; Skripuletz et al., 2008). Outside of the brain, mice treated with cuprizone also demonstrate liver dysfunction (Suzuki and Kikkawa, 1969). A standard treatment protocol of feeding 8-week-old C57BL/6 mice 0.2% cuprizone for 6 weeks has been shown to produce acute demyelination while minimizing hepatic toxicity and the mortality rate of the mice (Torkildsen et al., 2008).

There have in fact been few studies into cuprizone's effect on copper concentrations in brain tissue, and the literature does not provide conclusive evidence that cuprizone causes copper deficiency in the brain. An early study indeed found decreased copper levels in whole brains of Swiss mice fed a 0.5% cuprizone diet for 3–4 weeks (Venturini, 1973). Recent studies, however, have actually reported increased levels of copper in several brain regions following chronic cuprizone treatment in CD mice for 3, 6, and 9 months with 0.2% cuprizone administrated in drinking water (Zatta et al., 2005) and in C57BL/6 mice fed 0.2% cuprizone for 1 week (Tezuka et al., 2013). The discrepancies in the findings could be the result of differences in mouse strains, cuprizone administration, and the time course of administration, or a true

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misinterpretation of cuprizone's ability to reduce copper levels in the brain.

To further determine whether cuprizone's method of action is to reduce copper concentrations in brain tissue, we investigate in this paper copper concentrations in the blood, liver, kidneys, and brain regions of C57BL/6 male mice fed 0.2% cuprizone for 6 weeks compared to controls, as this has become a standard treatment regime for demyelination studies. We also investigate levels of manganese, iron, and zinc, since the homeostasis of these transition metals are linked (Garcia et al., 2006; Gunshin et al., 1997; Scheuhammer and Cherian, 1981) and it may be that changes in copper levels in the body may disrupt the levels of these other transition metals.

## 2. Materials and methods

### 2.1. Animal model

All experiments were approved by the Animal Research Ethics Board at McMaster University and were carried out in male C57Bl/6 mice (Jackson Laboratories, ME). Control ($n = 15$) and treated ($n = 15$) groups of mice were ordered at 7 weeks old and allowed to acclimatize for 1 week prior to onset of the experiment, such that the cuprizone treatments began when the animals were 8 weeks old. All animals were housed five to a cage, provided with food and tap water ad libitum and kept on a 12 h light/dark cycle. Cuprizone (Sigma–Aldrich, MO) was milled into 8640 Teklad 22/5 rodent chow (Harlan Laboratories Inc., WI) at a concentration of 0.2% dry chemical to dry food weight and formed into half-inch pellets. A treated group of mice was fed this 0.2% cuprizone diet for 6 weeks. A control group of mice were fed standard 8640 Teklad 22/5 Rodent Diet (Harlan Laboratories, Inc., WI). Treated animals were observed once daily and all animals were weighed weekly during treatment and sacrificed at end of the 6 week time-course. For euthanasia, each mouse was induced with 5% isofluorane in 100% oxygen to reach surgical level anesthesia. The thorax was cut open and blood was drawn from the left ventricle by cardiac puncture. Kidney and liver tissue samples were obtained by dissection. The animal was then decapitated and the brain was harvested intact. The whole brain was snap-frozen in isopentane cooled in a liquid nitrogen bath to prevent diffusion of metal ions throughout the tissue prior to dissection. Control and treated brains were partially thawed and the following regions were dissected: the cortex (sampled in the frontal region), the cerebellum (entire), thalamus (sampled in a medial region), the striatum (entire), and the hippocampus (entire). The wet weight of all samples was recorded. We were not able collect enough corpus callosum tissue for metal measurements, especially in the cuprizone-treated mice.

### 2.2. Histopathology

Pathology was investigated in three control and three treated animals. For whole-body perfusion, each animal was induced with 65 mg/kg body weight of sodium pentobarbital to reach surgical level anesthesia. The thorax was cut open and deflected and a 0.1 ml heparin sodium solution was injected into the left ventricle. A needle attached to a bag of lactated Ringers solution located 50 cm above the mouse was placed in the left ventricle. The right atrium was cut and the Ringers solution was allowed to flow through the animal, washing out the blood. After 5 min of flow, the solution was replaced with 10% phosphate-buffered formalin and allowed to flow for 6 more min. The brain was dissected and further fixed in formalin overnight. Coronal blocks of the brain were cut and dehydrated by a series of rising concentrations of alcohols from 50–100% and then xylene. The tissues were embedded in paraffin and 5 μm thick sections were cut, mounted on glass slides and costained with hematoxylin and eosin (H&E) and luxol fast Blue (LFB), a myelin stain. The slides with tissues were coverslipped and analyzed under a Nikon Eclipse 50i light microscope by a pathologist blinded to the treatment of individual mice. Representative areas of the corpus callosum, striatum, and cerebellum were photographed.

### 2.3. X-ray fluorescence (XRF) measurements

XRF was used to measure iron, copper, and zinc content in the tissue samples (Al-Ebraheem et al., 2009). Fresh tissue was mounted into XRF sample holders with the sample forming a disc 2 mm thick and 4 mm in diameter. The samples were kept frozen at −80 °C and allowed to thaw for a few minutes prior to the measurement. The X-ray source was a Molybdenum target tube. The output beam was monochromated to approximately 17.5 keV and focused on a sample size of 2 by 2 mm using a multi-layer X-ray optics device. Samples were mounted at 90° between the incident X-ray beam and the XRF detector and located 0.5 cm away from the XRF detector during data collection. Measurements were made with the X-ray tube operating at 50 kV and 500 mA with a counting time of 2,6100 s, or at 50 kV and 320 mA with a counting time of 52,200 s. The elements of interest (iron, copper, and zinc) were identified by the photopeaks associated with their K-alpha fluorescence photon emission at 6.4 keV and 8.04 keV, respectively.

To quantify the concentration of iron and copper in the tissue samples, calibration curves were constructed for each element. Calibration solutions with known concentrations of the elements (0–65 ppm iron, 0–50 ppm copper, 0–50 ppm zinc) were measured using the same procedure as the tissue samples. A linear relationship between the elemental quantity and ratio of fluorescence to scatter photopeak areas over the relevant concentration range was established. The linear calibration equations were used to quantify the iron, copper, and zinc concentrations in the tissue samples.

XRF spectra were analyzed using PeakFit spectrometry analysis software (PeakFitTM SPSS, Inc., AISN Software, Inc.). The fluorescence photopeaks were smoothed and the background was subtracted. A Gaussian function was used to fit the photopeaks and the net area of the peak was determined. The same procedure was applied to analyze the escape silicon peak, tail, Compton, and Coherent scatter peaks of every spectrum. The total scatter peak area was used as a normalization factor for the detected fluorescence photons. The ratio of fluorescence to scatter peak areas was then used to calculate the elemental concentrations in the samples.

The accuracy of the XRF method for quantification of copper, iron and zinc in tissue samples was validated using a standard reference material of the same weight as the samples (Table 1). This material was homogenized lobster (LUTS-1, "Non defatted lobster hepatopancreas reference material for trace elements," National Research Council, Canada) due to its material matrix of mainly water and lipid, similar to brain tissue.

#### 2.3.1. Neutron activation analysis (NAA)

NAA was used to measure the manganese content in the tissue specimens. Samples were mounted in polyethylene tubes and

**Table 1**
Certified and experimental concentrations of Cu, Fe and Zn in the LUTS-1, "Non-defatted lobster hepatopancreas reference material for trace elements," National Research Council, Canada.

| Element | Certified value (mg/kg) | Experimental value (mg/kg) |
|---|---|---|
| Cu | 15.9 ± 1.2 | 17.4 ± 1.4 |
| Fe | 11.6 ± 0.9 | 12.4 ± 2.5 |
| Zn | 12.4 ± 0.8 | 16.5 ± 3.4 |